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Inhibition by staurosporine of mitogen-induced calcium mobilisation in human T lymphoblasts

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Addition of monoclonal antibodies (UCHT1) directed against the antigen receptor/CD3 complex or the addition of the mitogens concanavalin A or phytohemagglutinin to human T lymphoblasts induced an elevation of the cytosolic free calcium concentration ($[Ca^{2+}]_i$) as monitored by the fluorescence of the intracellular fura-2. The rise in $[Ca^{2+}]_i$ induced by these agents was effectively inhibited by staurosporine, an agent which inhibits protein kinase (PK)C and PKA. Conversely the PKC activator 12-*O*-tetradecanoyl phorbol-13-acetate decreased the delay and accelerated the rate of elevation of $[Ca^{2+}]_i$ induced by the mitogens and UCHT1. Increasing the concentration of cAMP or cGMP in the T lymphoblasts had no effect on agonist-induced elevation of $[Ca^{2+}]_i$. Our data indicate that PKC can exert a positive feedback on the level of cytosolic Ca^{2+} in T lymphocytes, in contrast to what has been observed with other cells.

Ca^{2+}; Fura-2; Staurosporine; T lymphocyte

1. INTRODUCTION

T lymphocyte activation via the antigen receptor/CD3 complex results in elevation of cytosolic free calcium levels ($[Ca^{2+}]_i$) and protein kinase C (PKC) stimulation [1,2]. These two signals synergise to induce the expression of IL-2 receptors, IL-2 production and hence T cell growth. The initial increase in $[Ca^{2+}]_i$ is due to the mobilisation of intracellular Ca^{2+} and is apparently mediated by Ins 1,4,5-P_3, a product of the hydrolysis of phosphatidylinositol 4,5-bisphosphate [3–7]. The antigen receptor/CD_3 mediated elevation of $[Ca^{2+}]_i$ is then sustained by influx of extracellular Ca^{2+} probably through voltage-independent Ca^{2+} channels, which can also be activated by Ins 1,4,5-P_3 [3,8].

There is a requirement for a prolonged ongoing elevation of the $[Ca^{2+}]_i$ for T cell activation [2]. Consequently, it is predicted that feedback mechanisms that regulate ligand-mediated alterations in Ca^{2+} homeostasis would have profound effects on the T cell activation response. In this respect, PKC appears to have a feedback role in the T cell, since activation of PKC results in down-regulation of the surface expression of the antigen receptor/CD_3 complex [9–12]. Moreover, there are reports that activation of PKC inhibits the elevation of $[Ca^{2+}]_i$ induced by stimulation of the vasopressin and bombesin receptors on Swiss 3T3 cells and the thrombin receptor on human platelets [13,14]. However, other calcium-mobilising receptors such as the platelet-derived growth factor receptor in Swiss 3T3 cells are not inhibited by PKC [13].

The aim of the present study therefore was to determine the role of PKC in the modulation of T cell $[Ca^{2+}]_i$ following stimulation of the antigen receptor/CD3 complex.

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Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; Con A, concanavalin A; PHA, phytohemagglutinin; IL-2, interleukin 2; $[Ca^{2+}]_i$, cytosolic free calcium concentration; PKA(C), protein kinase A(C); Ins 1,4-P_2, inositol 1,4-bisphosphate; Ins 1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins 1,3,4,5-P_4, inositol 1,3,4,5-tetrakisphosphate

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2. EXPERIMENTAL

Peripheral blood mononuclear cells were separated by Ficoll-Paque (Pharmacia) gradient centrifugation of fresh human blood as described [15], from which human T lymphoblasts were prepared according to Smith and Cantrell [16].

T lymphoblasts were suspended at 10^7 cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and incubated for 30 min at 37°C with 2.5 μM fura-2 acetoxymethyl ester (fura-2/AM). After incubation, the cells were washed three times in 20 ml calcium-free, Hepes-buffered Tyrode solution and finally resuspended at 1×10^6 cells/ml in Hepes buffer, as in [17].

Aliquots of cells (1 ml) suspended in Hepes-buffered Tyrode solution were dispensed into disposable cuvettes and the external Ca^{2+} concentration ($[Ca^{2+}]_e$) adjusted to 1 mM with $CaCl_2$. Cells were allowed to equilibrate at 37°C for 2 min before drug addition to the cell-containing cuvettes. The $[Ca^{2+}]_i$ of T lymphoblasts was determined by fluorescence, as described in [17].

The following chemicals were obtained from the sources indicated: fura-2/AM from Molecular Probes (Eugene, OR); Con A from Sigma (Poole, England); PHA from Wellcome (Beckenham, England); staurosporine from Fluorochem (UK); UCHT1 was from Imperial Cancer Research Fund (London, England).

3. RESULTS

The antigen receptor/CD3 complex can be triggered by antibodies reactive with the CD3 antigen [1]. Thus, addition of the CD3 monoclonal antibody UCHT1 (2.4–24 μg/ml), or the polyclonal T cell mitogens Con A (3–300 μg/ml) and PHA (0.6–60 μg/ml) to fura-2-loaded human T lymphoblasts suspended in Hepes-buffered Tyrode solution containing 1 mM $[Ca^{2+}]_e$, produced elevation of $[Ca^{2+}]_i$ above basal levels of 166 ± 17 nM (n = 30) to approx. 200–2000 nM depending on dose (fig.1). As can be seen in fig.1, increasing the dose of UCHT1 or either mitogen reduces the delay in response, accelerates the rate of elevation and increases the peak calcium signal.

The construction of dose-response curves for each of the agonists, allowed the selection of sub-optimal doses of UCHT1 (8 μg/ml), Con A (30 μg/ml) and PHA (20 μg/ml), all of which elevated the $[Ca^{2+}]_i$ to approx. 600 nM. To examine the role of PKC, the T lymphoblasts were pretreated with staurosporine, an agent which inhibits PKC and PKA [18]. 1 min pre-treatment with staurosporine (9–300 nM) resulted in a dose-dependent inhibition of sub-optimal UCHT1, Con A and PHA-induced elevation of $[Ca^{2+}]_i$ (figs 1,2). The IC_{50} values for inhibition by staurosporine of sub-optimal UCHT1, Con A and PHA-induced elevation of $[Ca^{2+}]_i$ were 66 ± 18, 91 ± 7 and 82 ± 10 nM, respectively. A similar range of staurosporine concentrations (9–300 nM) had no effect on resting $[Ca^{2+}]_i$.

Conversely, incubation of the T lymphoblasts with the PKC activator TPA (0.48–48 nM) 10 min

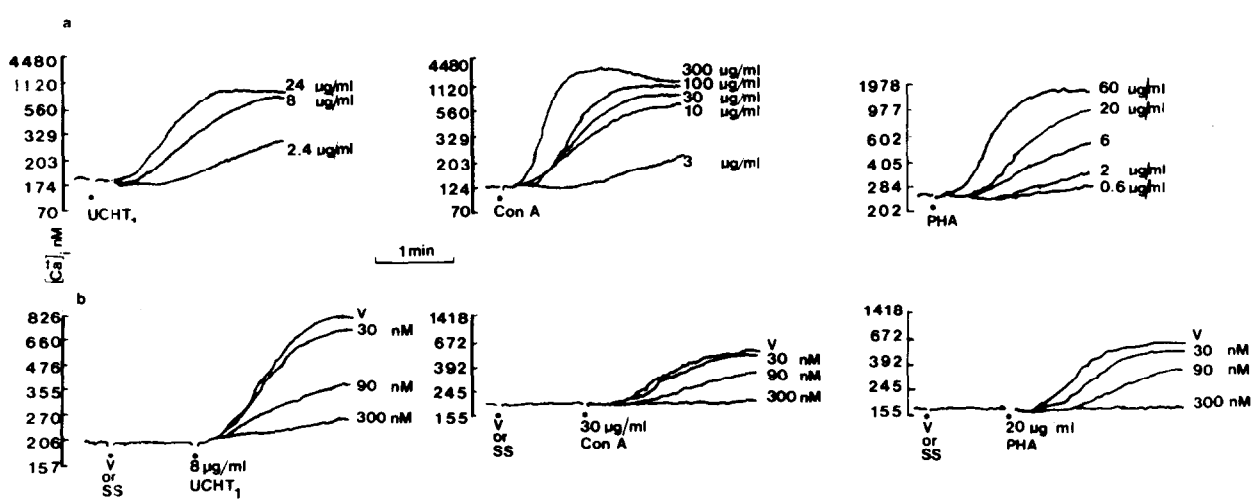

Fig.1. The effect of (a) UCHT1, Con A and PHA on T lymphoblast $[Ca^{2+}]_i$ and (b) 1 min pre-treatment with staurosporine on elevations of T lymphoblast $[Ca^{2+}]_i$ induced by suboptimal UCHT1 (8 μg/ml), Con A (30 μg/ml) and PHA (20 μg/ml). The non-linear vertical scale is the result of transforming fluorescent output to T lymphoblast $[Ca^{2+}]_i$. The traces were obtained from one experiment but are representative of four other experiments.

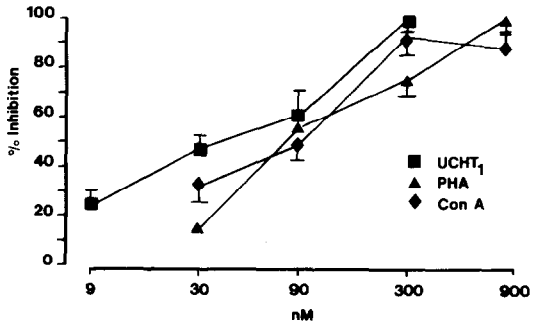

Fig.2. Dose-response curves of inhibition by 1 min pre-treatment with staurosporine of elevations of T lymphoblast $[Ca^{2+}]_i$ induced by UCHT1 (8 μg/ml), Con A (30 μg/ml), and PHA (20 μg/ml). Each point represents the mean ± SE of determinations performed in four separate experiments.

prior to the addition of UCHT1 or Con A resulted in a decreased delay, accelerated rate of elevation and an enhanced peak calcium signal compared to TPA vehicle-treated cells ($p < 0.05$; fig.3a–c). Furthermore, pre-treatment of T lymphoblasts for 10 min with another PKC activator, phorbol 12,13-dibutyrate (50 nM), produced an essentially similar enhancement of ligand-induced elevation of $[Ca^{2+}]_i$ compared to vehicle-treated cells ($p < 0.05$, $n = 5$; not shown). In contrast, pre-treatment of the T lymphoblasts with 4β-phorbol (4.8–480 nM, $n = 4$) which does not activate PKC, did not modify sub-optimal UCHT1 or Con A-induced elevation of $[Ca^{2+}]_i$.

To lend support to the notion that staurosporine was inhibiting PKC in these studies, TPA pretreatment prior to the addition of staurosporine resulted in a reduction of the inhibitory effect of staurosporine on PHA-induced $[Ca^{2+}]_i$ elevation (fig.3d).

To examine the possibility that the staurosporine effects could be mediated by cAMP- or cGMP-dependent kinases, the following experiments were performed to determine the role of cAMP and cGMP in the modulation of T cell $[Ca^{2+}]_i$. The cells were pretreated for 1–10 min with either forskolin (10–100 μM), an adenyl cyclase activator; or 8-bromo-cAMP (50 μM), a cell-permeant cAMP analog; or sodium nitroprusside (30–150 μM), a guanyl cyclase activator; or 8-bromo cGMP (50 μM). These compounds had no significant ef-

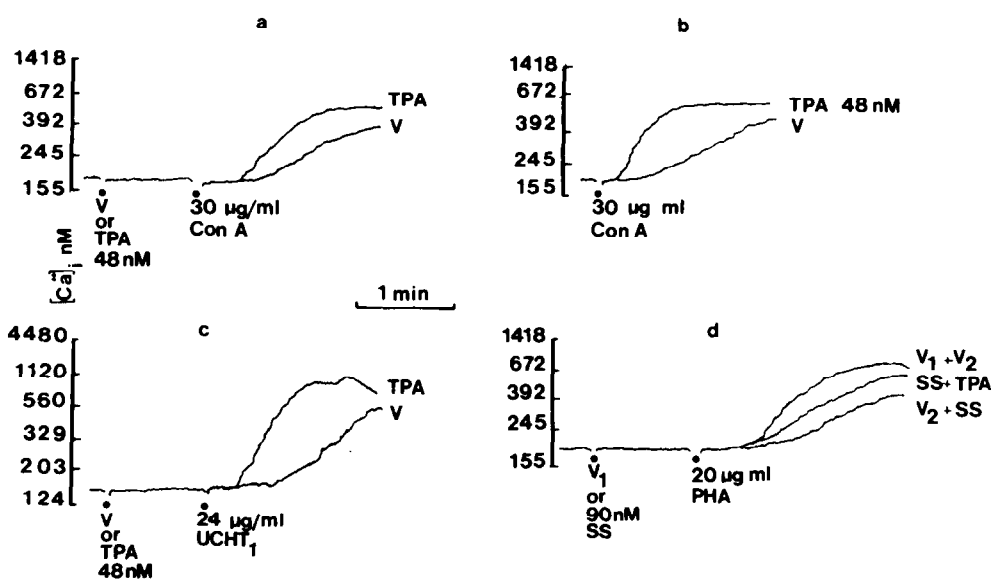

Fig.3. Effect of 1 min (a) or 10 min (b) pretreatment with vehicle or TPA (48 nM) on suboptimal Con A (30 μg/ml)-induced elevation of T lymphoblast $[Ca^{2+}]_i$; (c) effect of 1 min pretreatment with vehicle or TPA (48 nM) on suboptimal UCHT1 (24 μg/ml)-induced elevation of T lymphoblast $[Ca^{2+}]_i$; (d) effect of pretreatment for 1 min with staurospermine vehicle (V_1) and for 2 min with TPA vehicle (V_2), or with 90 nM staurosporine (SS) and V_2, or with SS and 48 nM TPA on PHA (20 μg/ml)-induced elevation of T lymphoblast $[Ca^{2+}]_i$. *The non-linear vertical scale is the result of transforming fluorescent output to T lymphoblast* $[Ca^{2+}]_i$. The traces were obtained from one experiment but are representative of four other experiments.

fect ($p > 0.05$) on sub-optimal UCHT1 and PHA-induced elevation of $[Ca^{2+}]_i$. Accordingly, staurosporine effects on cAMP- or cGMP-dependent kinases are unlikely to influence T cell $[Ca^{2+}]_i$.

4. DISCUSSION

The present data indicate a positive feedback role for PKC in the regulation of $[Ca^{2+}]_i$ in T lymphocytes, since activation of PKC enhances, while inhibition of PKC prevents ligand-induced elevation of $[Ca^{2+}]_i$. The marked enhancement of receptor-operated elevation of $[Ca^{2+}]_i$ via PKC is surprising as PKC stimulation immediately increases the rate of internalisation of the antigen receptor/CD3 complex, thereby decreasing its surface levels [9–12]. Thus, PKC activation appears to produce a considerable facilitation of the calcium elevation that occurs via stimulation of the remaining cell surface located receptors.

A positive feedback role for PKC in the modulation of cytosolic calcium appears unique to T lymphocytes, since stimulation of PKC in platelets, polymorphonuclear cells, leukemic basophils, pituitary cells, PC12 cells, hepatocytes or B lymphocytes (for references see [14,19]) inhibits receptor-operated elevation of $[Ca^{2+}]_i$. In platelets the inhibitory effect of PKC on the calcium-mobilising activity of receptors is possibly explained by a PKC-induced reduction in Ins 1,4,5-P_3 levels via at least two mechanisms: inhibition of phospholipase C [20] and thus reduced Ins 1,4,5-P_3 formation and also increased conversion of Ins 1,4,5-P_3 to Ins 1,4-P_2 [21].

It remains to be determined therefore whether the effects of PKC on T cell intracellular calcium are explained by its effects on the metabolism of phosphoinositides. However, there are reports that PKC can stimulate the PI cycle to produce increased levels of phosphatidylinositol 4,5-bisphosphate [22]. In addition, Imboden and Pattison [23] have recently demonstrated that PKC stimulation leads to an increased conversion of Ins 1,4,5-P_3 to Ins 1,3,4,5-P_4. Although the latter does not mobilise intracellular calcium, there is evidence to suggest that Ins 1,3,4-P_3 and Ins 1,3,4,5-P_4 work in concert to promote influx of calcium across the plasma membrane [24]. It is tempting to speculate that this could be the mechanism of the enhanced elevation of $[Ca^{2+}]_i$ reported in the present study.

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